Gastroenterologia Japonica
Vol. 18, No. 4
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Printed in Japan

—Original Article—

HEPATOCYTE PLASMA MEMBRANE ANTIGENS. II. CHARACTERIZATION OF LIVER-SPECIFIC MEMBRANE LIPOPROTEIN (LP-1) AND TAMM-HORSFALL GLYCOPROTEIN (THGP) LIKE ANTIGENS (HEPATIC THGP) ON THE PLASMA MEMBRANE OF CHANG LIVER CELL

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Summary

Radioiodinated cell-surface antigens of Chang liver cells recognized with anti-liver-specific membrane lipoprotein (anti-LP-1) and anti-Tamm-Horsfall glycoprotein (anti-THGP) rabbit antibodies were analyzed by the immunoprecipitation followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The analysis revealed that the antigens precipitated with anti-LP-1 were heterogeneous in molecular species consisting of 2 major and 5 minor polypeptides with molecular weights ranging from 36×10^3 (36 K) to 250 K daltons. That with anti-urinary THGP was a single polypeptide with a molecular weight of 67 K daltons, which was different from urinary THGP in molecular size, and was designated hepatic THGP.

Key Words: *Chang liver cell, liver-specific membrane lipoprotein (LP-1), Tamm-Horsfall glycoprotein (THGP) like antigen.*

Introduction

Liver-specific membrane lipoprotein (LP-1)[1], an antigen complex in the hepatocyte cell membrane[2-4], and Tamm-Horsfall glycoprotein (THGP)[5], a carbohydrate-rich urinary glycoprotein[6], have been investigated in relation to immune responses in liver diseases[7-10]. Antibodies reacting with LP-1 and THGP have been detected in the sera of patients with acute hepatitis and chronic active hepatitis by radioimmunoassay[7,8] and antibody-dependent cell-mediated cytotoxicity (ADCC) assay[10], where the serum level of the antibodies correlated with the severity of liver damage. On the other hand, antigens reacting with anti-LP-1 and anti-THGP rabbit antibodies were recognized

Received February 18, 1983. Accepted April 4, 1983.

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We wish to thank the staff in the RI research center of Okayama University Medical School for their technical assistance.

This research was supported in a part by a research grant from the Scientific Research Fund (1982, No. 57770490) of the Ministry of Education, Science and Culture of Japan.

on sections of normal liver as membrane fluorescence outlining hepatocytes by indirect immunofluorescence staining[4,9]. Furthermore, anti-LP-1 rabbit antibodies were found to bind to cultured normal human hepatocytes, Chang liver cells[11]. In a previous study[12], we demonstrated that both anti-LP-1 and anti-THGP rabbit antibodies bound to Chang liver cells, by immunofluorescence staining and ^{125}I-protein A binding assay. However, the molecular species of the antigens on the hepatocyte plasma membrane recognized with these antibodies remained unknown.

Radioiodination of cell-surface proteins with lactoperoxidase, solubilization, immunoprecipitation and the following analysis of the antigens with polyacrylamide gel electrophoresis (PAGE) has been proved to be a useful technique to study cell surface antigens[13]. In this study, cell-surface proteins of Chang liver cells were radioiodinated, and the radioiodinated cell-surface antigens recognized with anti-LP-1 and anti-THGP rabbit antibodies were analyzed by sodium dodecyl sulfate-polyacrylamide gel electroresis (SDS-PAGE) after the immunoprecipitation.

Materials and Methods

Preparation of anti-LP-1 and anti-THGP rabbit antiserum: LP-1 was prepared from normal human autopsy liver tissue by the method of McFarlane et al.[2] and THGP from pooled normal urine by the method of Fletcher[6]. Anti-LP-1 and anti-THGP rabbit antiserum were prepared as previously described[12].

Cell and culture: Chang liver cells[14] were used in the present study. The cells were grown in Eagle's minimum essential medium (MEM; GIBCO Lab., New York) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO Lab.), 100 U/ml penicillin and 100 μg/ml streptomycine at 37°C in humidified 95% air-5% CO_2.

Immunofluorescence staining: Chang liver cells were examined for reactivity with anti-LP-1 and anti-THGP rabbit antiserum by indirect immunofluorescence staining with fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit γ-globulin goat immunoglobulins (Boehringer-werke AG.). Indirect immunofluorescence staining was performed as previously described[12]. Briefly, Chang liver cells grown on a cover glass were incubated with the antiserum diluted 10-fold for 45 min. After the incubation, the cells were washed with Dulbecco's modified phosphate buffered saline (PBS; GIBCO Lab.) and incubated with FITC-conjugated anti-rabbit γ-globulin for further 45 min. After another wash with PBS, the cells on the cover glass were examined for membrane fluorescence by an ultraviolet microscopy (OLYMPUS, model BH-RFL). A control test was run with normal rabbit serum instead of rabbit antisera.

^{125}I-Protein A binding assay: Antibodies bound to Chang liver cells were determined by ^{125}I-protein A binding assay. The ^{125}I-protein A binding assay was performed as previously described[12]. Briefly, Chang liver cells grown in a microtestplate (Falcon 3040, Div. Becton, Dickinson and Co. California) were incubated with test serum diluted with PBS containing 1% bovine serum albumin (BSA) for 45 min. After incubation, the cells were washed with PBS and incubated with 25 μl ^{125}I-protein A solution (approximately 50,000 cpm) for further 45 min. The cells were again washed and lysed by adding 150 μl of 1 N NaOH. Radioactivity in cell lysate was counted by a gamma counter (Aloka, model ARB-500, Tokyo). The percentage of ^{125}I-protein A bound to the cells was culculated according to the following formula.

Percentage of ^{125}I-protein A bound = {(cpm in cell lysate treated with test serum − cpm in cell lysate treated with PBS containing 1%

BSA)/cpm of ^{125}I-protein A added} × 100.

The assay was done in duplicate. The viability of the cells during the experiment was greater than 90% as determined by a trypan blue dye exclusion test.

Cell culture on microcarrier beads: Chang liver cells were cultivated on microcarrier beads (Cytodex-I, Pharmacia Fine Chemicals, Uppsala, Sweden). The monolayer-culture cells were harvested by 0.25% trypsin in PBS and 4 × 10^6 cells were suspended in 10 ml culture medium with 10% FCS in a culture tube (Falcon 3033). One ml of 2% (w/v) Cytodex-I in the growth medium was thereafter added to the tube. The cells were cultivated for 3 days at 37°C with continuous stirring on a rocker platform (Bellco Glass. Inc. New Jersey). The medium was changed daily.

Radioiodination and solubilization of radioiodinated cell-surface proteins: Cell-surface proteins of Chang liver cells were radioiodinated by the method of Haustein[15], with a minor modification. Cells on the microcarrier beads were washed 5 times with 10 ml PBS. The pellet of the beads, collected by standing the tube for 2 min, was mixed with 150 μl of PBS, 10 μl of Na ^{125}I (100 mCi/ml; New England Nuclear) and 10 μl lactoperoxidase solution (75 U/ml). The reaction was initiated by adding 10 μl 0.03% H_2O_2. The suspension was subsequently agitated gently and stood for 4 min at 4°C. The reaction was repeated three times by adding 10 μl 0.03% H_2O_2 at 4 min intervals. The reaction was stopped by adding 10 ml ice cold PBS and the microcarrier beads were washed 5 times with PBS. Radioiodinated cells were then incubated with 1 ml of 1% Triton X-100 in NET-buffer (0.1 M Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetate, EDTA, 0.15 M NaCl) containing 2 mM phenylmethy-sulphonyl-fluoride (PMSF) for 15 min on ice. The solubilized material was collected by centrifugation at 1500×g for 5 min and clarified by further centrifugation at 10,000×g for 60 min at 4°C. The clarified supernatant was used for the immunoprecipitation described below. Radioactivity in the supernatant was counted with the gamma counter.

Immunoprecipitation: One hundred microliters of the solubilized material (93 × 10^4 cpm/100 μl) were mixed with 100 μl anti-LP-1 or anti-THGP rabbit antiserum diluted 10-fold and rotated overnight at 4°C. After the incubation, 100 μl of 10% (w/v) protein A-sepharose CL-4B (Pharmacia Fine Chemicals) in NET-buffer containing 0.5% BSA and 0.05% Triton X-100 were added to the reaction tube, and incubated for a further 4 hours at room temperature in order to coprecipitate the antigen-antibody complexes. Then the precipitates were washed 5 times with 0.05% Triton X-100 in NET-buffer and stored at −20°C until used. The radioactivities in the precipitates were determined with the gamma counter. The antigen-antibody complexes bound to the protein A-sepharose CL-4B were suspended in 60 μl of the sample buffer (0.065 M Tris-HCl, pH 6.8, 10% glycerin, 3% SDS, 5% 2-mercaptethanol) and heated at 100°C for 3 min. The samples were centrifuged at 1000×g for 2 min, and 50 μl of the supernatants were applied to SDS-PAGE.

SDS-PAGE and autoradiography: SDS-polyacrylamide gel electrophoresis was performed as described previously[16] with a minor modification. Electrophoresis was performed with bromophenol blue as a tracking dye in 7.5% acrylamide gel for 3 hours. The gel thickness was 0.75 mm and the current applied was 30 mA/plate. The gels were stained with Coomassie brilliant blue R-250[17] or the silver staining[18].

The molecular weights of cell-surface antigens were determined by parallel electrophoresis of molecular weight calibration marker proteins (Pharmacia Fine Chemicals), which con-

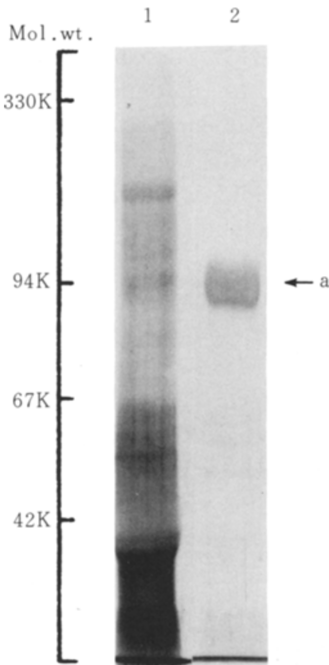


Fig. 1. Analysis by SDS-PAGE of liver-specific membrane lipoprotein (LP-1) prepared from normal liver tissue (1) and Tamm-Horsfall glycoprotein (THGP) from normal urine (2).

sisted of thyroglobulin (330 × 10^3 daltons, 330 K), phosphorylase B (94 K), bovine serum albumin (67 K), ovalubumin (42 K), and carbonic anhydrase (30 K).

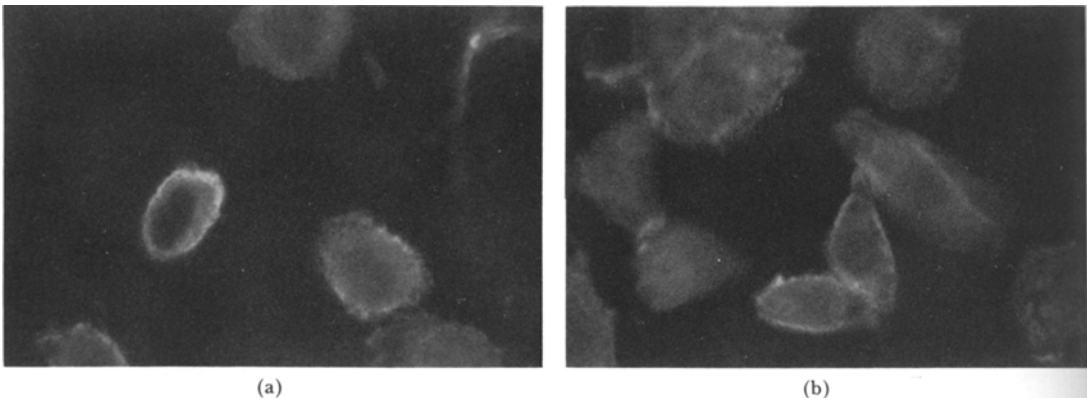
Autoradiography was performed by exposing the dried gel to a X-ray film (Kodak X-Omst AR film) for 10 days at −70°C.

Results

Anti-LP-1 and anti-THGP antibodies bound to cells: Rabbit antisera against LP-1 or THGP were prepared as described in the Materials and Methods. LP-1 prepared from normal liver tissue and THGP from normal urine, which were used as antigens for the immunization, were analyzed by SDS-PAGE. The staining patterns of the gel are shown in **Fig. 1**. LP-1 consisted of many diffuse bands, while THGP was one major band (arrow a) with a molecular weight of 104 K daltons. The antisera raised in rabbits gave at least two precipitin lines between anti-LP-1 and LP-1, and one between anti-THGP and THGP in an immunodiffusion test.

Indirect immunofluorescence staining with either anti-LP-1 or anti-THGP rabbit antiserum showed linear patterns of membrane fluorescence on Chang liver cells (**Fig. 2a** and **2b**), that with normal rabbit serum was negative for fluorescence.

Anti-LP-1 and anti-THGP rabbit antibodies bound to Chang liver cells were also determined semiquantitatively by the ^{125}I-protein A binding assay (**Fig. 3**). The radioactivity of ^{125}I-



(a) (b)

Fig. 2. Indirect immunofluorescence of Chang liver cells after incubation with anti-LP-1 (a) or anti-THGP (b) rabbit antiserum. (Magnification ×400)

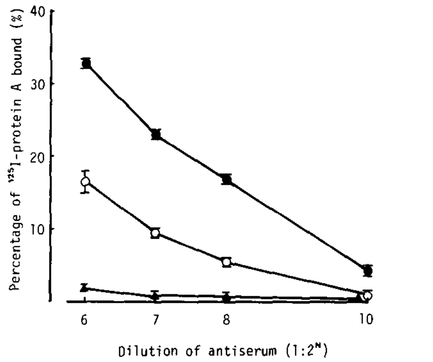


Fig. 3. ^{125}I-protein A binding assay. Chang liver cells were incubated with serially diluted anti-LP-1 (●—●), anti-THGP (○—○) rabbit antiserum or normal rabbit serum (▲—▲). Antibodies bound to cells were examined by the ^{125}I-protein A binding assay as described in Materials and Methods.

protein A bound to Chang liver cells after pre-incubation with either anti-LP-1 or anti-THGP antiserum was higher than that with normal rabbit serum. The percentage of ^{125}I-protein A bound to the cells decreased in proportion to the amount of antiserum added.

Immunoprecipitation of membrane LP-1 and THGP: The cell-surface antigens on Chang liver cells precipitated with the anti-LP-1 or anti-THGP rabbit antibodies were analyzed. Chang liver cells were externally radioiodinated, solubilized and incubated with the test sera. The immune complexes formed during incubation were coprecipitated with protein A-sepharose CL-4B. The radioactivities of the precipitates are shown in **Table 1**. The radioactivities of the immunoprecipitates treated with anti-LP-1 or anti-THGP rabbit antiserum were higher than that with normal rabbit serum.

Demonstration of cell-surface antigens recognized with anti-LP-1 or anti-THGP rabbit antibodies: The antigens precipitated with the antisera were analyzed by SDS-PAGE as described in the Materials and Methods. The gel containing ^{125}I-labelled cell-surface antigens was dried and exposed to a X-ray film for the autoradiography. **Fig. 4** shows the autoradiographic patterns obtained. The antigens precipitated with anti-LP-1 contained at least 7 specific radioactive bands when compared with those with normal rabbit serum. The molecu-

Table 1. Radioactivity in immunoprecipitate

Test serum	Count in precipitate (cpm)
Normal rabbit serum	4420
Anti-LP-1	17790
Anti-THGP	8470

Solubilized radioiodinated cell-surface antigens of Chang liver cells (93 × 10^{4} cpm/100 μl) were incubated with anti-liver specific membrane lipoprotein (anti-LP-1), anti-Tamm-Horsfall glycoprotein (anti-THGP) rabbit antiserum or normal rabbit serum diluted 10-fold. The immune complexes formed in the incubation were coprecipitated with protein A-sepharose CL-4B. Radioactivity bound to protein A-sepharose CL-4B was counted with a gamma counter.

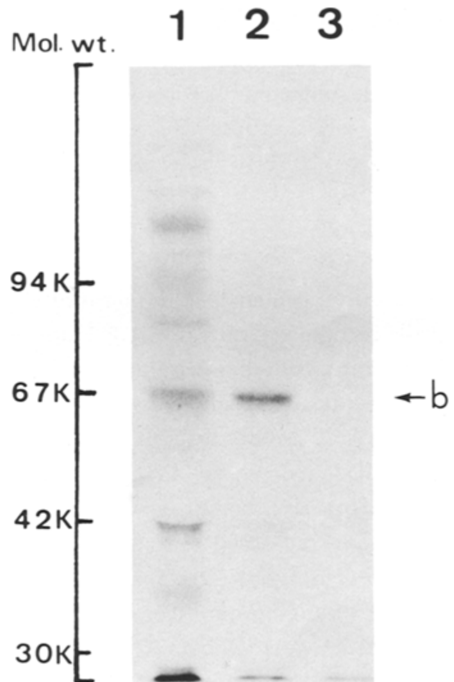


Fig. 4. Autoradiograph of the radioiodinated cell-surface antigens of Chang liver cells recognized with anti-LP-1 (1), anti-THGP (2) rabbit antiserum. A control test was run with normal rabbit serum (3).

lar weights of these antigens were in the range from 36 K to 250 K daltons. The 2 major bands were detected at 67 K and 140 K daltons, the 5 minor bands were at 36 K, 100 K, 115 K, 185 K and 250 K daltons. In the column of the sample treated with anti-THGP anti-serum, a specific band was given at the molecular weight of 67 K (arrow b). A radioactively labelled band with the same mobility was also observed in the column of the sample treated with anti-LP-1 antiserum. This band was not detected in the column of the sample treated with normal rabbit serum. These analyses revealed that the cell-surface antigens of Chang liver cells recognized with anti-LP-1 were heterogeneous in molecular species, while that with anti-THGP was a single molecular species with molecular weight of 67 K daltons, which was different from urinary THGP in molecular size (**Figs. 1** and 4).

Discussion

In this study, cell-surface antigens of Chang liver cells recognized with anti-LP-1 and anti-THGP rabbit antibodies were analyzed by immunoprecipitation and SDS-PAGE.

Tsantoulas et al.[9] found abnormal immune responses of leukocyte to THGP in patients with chronic active hepatitis associated with renal tubular acidosis. In addition, they demonstrated antigens cross-reacted with urinary THGP on the human liver cell membrane of a section of normal liver[9]. In a previous study, we showed that anti-THGP rabbit antibodies bound to Chang liver cells and PLC/PRF/5, human cell lines of hepatocyte and hepatoma cell, by an immunofluorescence staining and the ^{125}I-protein A binding assay[12]. However, the molecular species of the material recognized with anti-THGP has not been reported. The present analysis of the antigens by SDS-PAGE and the following visualization by the autoradiography, revealed that the antigen on Chang liver cells recognized with anti-THGP rabbit antibodies was a 67 K dalton molecule. This molecule was different from urinary THGP in molecular size, the latter having a molecular weight of 104 K daltons. The band of 67 K daltons was also seen in the LP-1 column (**Fig. 4**). Cochrane et al.[19] reported that there was no cross-reaction on immunodiffusion between LP-1 and anti-THGP. On the other hand, it was reported by another researcher that the absorption of anti-THGP with LP-1-coated cells was able to reduce the precent cytotoxicity of anti-THGP in ADCC assay using THGP-coated cells[10]. The question whether the molecular species recognized with anti-THGP was identical with that with anti-LP-1 or not, remains obscure since the bands appeared at 67 K daltons in the autoradiogram of LP-1 and THGP (**Fig. 4**) could be different molecules with the same molecular weight. IN is obvious that the molecule was not albumin, though the molecular weight was the same as that of human albumin. The reasons are that 1) Chang liver cells do not produce albumin in the medium, 2) fetal calf albumin was fully washed from the culture medium prior to the radioiodination and 3) anti-THGP rabbit antiserum used was found to not react immunologically with either BSA or human albumin.

LP-1 prepared from normal liver tissue had a heterogeneous molecular character, as shown in the analysis by SDS-PAGE (**Fig. 1**). McFarlane et al.[2] previously reported that LP-1 analyzed by SDS-PAGE showed 8 major and 5 minor components. Behrens et al.[4] also, analyzed LP-1 by this method, and demonstrated at least 8 distinct bands with molecular weights ranging from 40 K to 96 K daltons. LP-1 antigens on the plasma membrane of Chang liver cell have been demonstrated by immunofluorescence staining[11]. It is reported in this study, that the cell-surface antigens immunoprecipitated with the anti-LP-1 antiserum on

Chang liver cells were found to be heterogeneous in molecular character and contained 2 major and 5 minor proteins with molecular weights ranging from 36 K to 250 K daltons.

In this paper, it was shown that Chang liver cells expressed the antigens on their plasma membrane, with molecular weights of 36 K, 67 K, 100 K, 115 K, 140 K, 185 K and 250 K daltons, which were cross-reacted with LP-1 prepered from human liver homogenate, and an antigen 67 K daltons, which was cross-reacted with urinary THGP and designated hepatic THGP.

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